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Protection against Endotoxic Shock and Lipopolysaccharide-Induced Local Inflammation by Tetracycline: Correlation with Inhibition of Cytokine Secretion

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Septic shock results from excessive stimulation of host immune cells, particularly monocytes and macrophages, by lipopolysaccharide (LPS) released from gram-negative bacteria. Macrophage-derived cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β), have been identified as central mediators in the pathogenesis of septic shock and the resultant mortality. Therefore, these cytokines were targets for experimental therapy for septic shock. Because of tetracycline's ability to intervene in cellular mechanisms involved in cytokine secretion, we tested the effect of tetracycline on LPS-induced septic shock and inflammatory lesions in mice. Tetracycline was found to protect mice against LPS-induced lethality and to abolish clinical signs of LPS-induced inflammatory lesions. This protection correlates with tetracycline's ability to reduce LPS-induced TNF-α levels in serum. Furthermore, tetracycline was found to inhibit LPS-induced TNF-α and IL-1β secretion, but not cytokine mRNA accumulation, in human monocytes in vitro. The results presented here suggest that tetracycline is a potent drug for LPS-induced pathology and that its mechanism of action involves blockage of posttranscriptional events of cytokine production.

Septic shock is one of the leading causes of death in hospitalized patients, and mortality rates of up to 50% have been reported (6, 7, 33). Despite all efforts, no regimen today seems to be successful in the treatment of septic shock (3). The shock state results from a systemic infection with gram-negative bacteria (sepsis), but the clinical outcome of the infection leading to septic shock results primarily from the excessive stimulation of the host immune cells, particularly monocytes and macrophages, by lipopolysaccharides (LPS) (6, 35, 36). LPS is a major component of the outer cell wall of gram-negative bacteria, and it is the most potent stimulator of monocyte and macrophage cytokine secretion (36). Injection of LPS into the bloodstream results in pathophysiological changes that are similar to those seen in sepsis in experimental animals (22, 30, 39) as well as human volunteers (20, 25, 26, 28, 45).

Monocyte-derived inflammatory cytokines, particularly tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1), have been implicated in the pathogenesis of septic shock (8–10, 14, 18, 22, 24, 27, 30, 31, 37, 39, 44, 50). After injection of LPS, there is a rapid increase in serum TNF-α and IL-1 levels, which peak after 1 to 3 h. This response has similar kinetics in a variety of mammals, including humans (20, 22, 25, 26, 28, 30, 37, 39, 45). In addition, intravenous injection of TNF-α and/or IL-1β was found to result in a septic-shock-like state (2, 13, 32, 49).

Tetracyclines are a well-known family of antibiotics which are active against a wide range of gram-positive and gram-negative bacteria. In addition, anti-inflammatory properties of tetracycline, which are independent of its antibacterial activity, have been described, including the inhibition of protein kinase C and metalloproteinases (15, 19, 52). On the basis of the anti-inflammatory properties of tetracycline, the study presented here was designed to test the hypothesis that tetracycline can be used to prevent LPS-induced pathology and to inhibit cytokine secretion from human monocytes.

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MATERIALS AND METHODS

Endotoxic shock mouse model. Sabra mice (female, 6 to 7 weeks old, ≈35 g) were injected with *Salmonella typhosa* LPS (Sigma, St. Louis, Mo.) intravenously as a model for endotoxic shock. LPS was dissolved in a sterile pyrogen-free saline solution and dispersed by brief sonication. Experimental animals were given 1 ml of a 2-mg/ml tetracycline–HCl solution (58 mg/kg of body weight; Teva, Jerusalem, Israel) by gavage 20 min prior to intravenous LPS injection. Tetracycline administration was repeated 6 and 24 h after the LPS injection but at half of the original dose. Control animals received saline. Mouse mortality was monitored twice daily for 72 h, and in some experiments monitoring was continued once daily for up to 3 weeks.

For the determination of TNF-α levels in serum, mice were challenged with 500 μg of LPS intravenously. Simultaneously, 1 ml of the 2-mg/ml tetracycline–HCl solution was given by gavage to experimental animals, while control animals received saline. The animals were bled from the infraorbital plexus 2 h after LPS challenge, and the levels of TNF-α in the serum were determined by two-site enzyme-linked immunosorbent assay (ELISA) with anti-mouse TNF-α antibodies from Pharmingen (San Diego, Calif.) according to the manufacturer's instructions.

LPS-induced subcutaneous inflammatory lesions. LPS (from *Porphyromonas gingivalis*; 0.1 mg/0.1 ml per animal) was injected subcutaneously on the dorsa of Sabra or BALB/c mice. Simultaneously, the animals were injected intraperitoneally (i.p.) with 0.1 ml of a tetracycline solution (5 to 20 mg/ml) or saline. In some experiments, two groups of control animal were used; one received ampicillin (15 mg/ml; Sigma) i.p. and the other received saline. Lesion size was monitored daily for 3 weeks.

Assessing tetracycline's effect on TNF-α and IL-1β production by LPS-stimulated human monocytes. Fresh human monocytes were isolated from the buffy coats of healthy donors' blood specimens received from the blood bank of Hadassah Medical Center as previously described (42). Briefly, the buffy coats were fractionated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) sedimentation. The mononuclear cell fraction was removed, and the cells were washed three times and resuspended in RPMI 1640 medium supplemented to final concentrations of 100 U of penicillin per ml, 100 μg of streptomycin per ml, 2 mM L-glutamine (all from Biological Industries, Beit-Haemek, Israel), and 2% inactivated human type AB serum (Sigma). The cells were plated in 24-well culture plates at a concentration of 4×10^6 cells per well and incubated for 90 min. Nonadherent cells were removed by aspiration, and the wells were washed three times with phosphate-buffered saline. The adherent cells were then incubated with LPS with or without tetracycline. The medium was collected 18 h after LPS stimulation and kept at −70°C until assayed. TNF-α and IL-1β were assayed in the culture supernatants by two-site ELISA as previously described (42) but

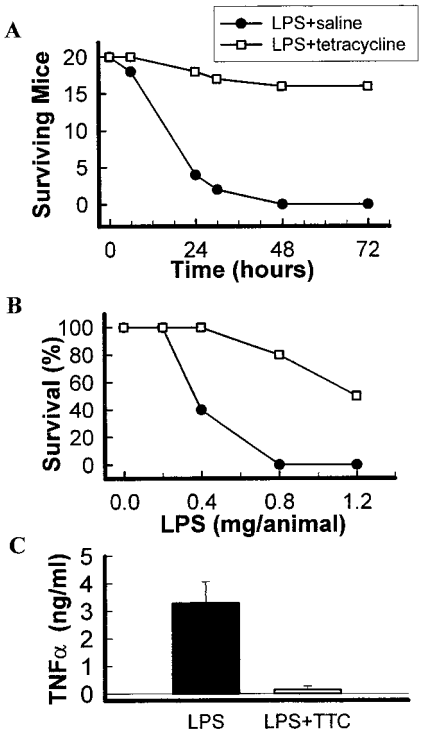

FIG. 1. (A) Survival of mice treated with tetracycline while in LPS-induced septic shock. Mice (n = 20 in each treatment group) were treated as described in Materials and Methods. Mouse mortality was monitored twice daily for at least 72 h. No further mortality was observed between day 3 and day 21 of follow-up. The two treatment groups were significantly different ($P < 0.01$). The same results were observed in three additional experiments, with five mice in each treatment group. (B) Survival of mice treated with tetracycline as described above and challenged with various doses of LPS. Lethality was assessed 48 h later. Each datum point represents 5 to 10 mice. (C) Effect of tetracycline (TTC) treatment on serum TNF-α levels in LPS-injected mice. Mice (n = 3 in each group) were challenged with LPS as described in the text. Animals were bled 2 h after LPS challenge, and the levels of TNF-α in the serum were determined by two-site ELISA. The results are means ± standard errors. The two groups are significantly different ($P = 0.016$, Student's t test).

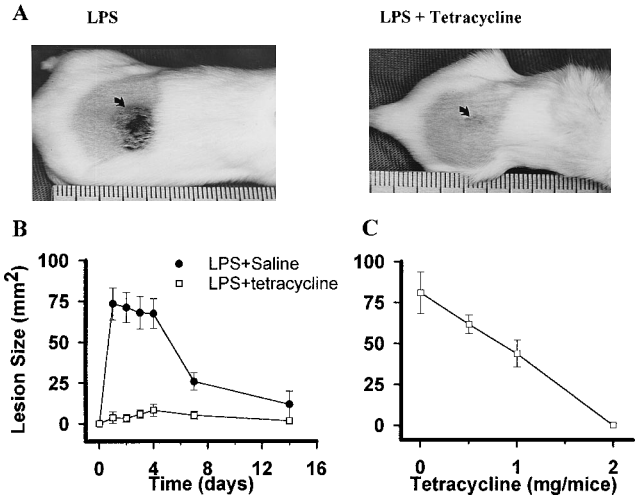

FIG. 2. Effect of tetracycline on LPS-induced lesions in mice. Mice were challenged with LPS as described in the text. Simultaneously, tetracycline was injected i.p. Tetracycline administration was repeated daily for 4 days. Lesion size was monitored daily. (A) Clinical views of the lesion (arrows). The left panel shows a sham-treated mouse with a typical LPS-induced lesion; 75 to 100% of the tetracycline (1.5 mg/day)-treated mice showed no lesion formation. The remaining animals developed minimal-sized lesions like the one illustrated in the right panel. Photographs were taken 72 h after LPS injection. (B) Effect of tetracycline on development of LPS-induced lesions. Mice (n = 5 in each group) were treated with either 1.5 mg of tetracycline per animal per day or saline and were followed up for 14 days. The two groups showed significantly different responses ($P < 0.01$). Similar results were obtained in three more experiments. (C) Dose-dependent inhibition of LPS-induced lesions by tetracycline. Mice (n = 5 in each group) were treated with various doses of tetracycline. Lesion size was measured after 48 h.

with antibodies from R&D Systems (Minneapolis Minn.). TNF-α, IL-1β, and β-actin mRNA were semiquantified by reverse transcription-PCR 2 h after LPS stimulation, as previously described (42, 46), with primers from Clontech (Palo Alto, Calif.) and from Strategene (La Jolla, Calif.) for IL-1β and for TNF-α and β-actin, respectively.

RESULTS

Tetracycline administered by gavage was found to protect mice against a lethal challenge with LPS (Fig. 1A). None of the mice that received 0.8 mg of LPS survived after 48 h, while 80% of the tetracycline-treated mice survived ($P < 0.01$, Z test with the Yates correction). Mice that survived the first 48 h recovered completely and continued to survive for at least another 3 weeks. In addition to significantly reducing mortality under these conditions, tetracycline treatment was found to shift the dose-response curve for LPS-induced lethality to the right (Fig. 1B). Complete protection by tetracycline was observed when the dose of LPS causing 60% mortality was used. Tetracycline administered by gavage to sham-challenged mice did not have a toxic effect.

Oral administration of tetracycline was also found to significantly reduce serum TNF-α levels in LPS-injected mice (Fig. 1C). While the TNF-α level in sera of LPS-challenged control mice was 3.28 ± 0.78 ng/ml (mean ± standard error), the level in tetracycline-treated mice was reduced to 0.14 ± 0.03 ng/ml (n = 3 for each group; $P = 0.016$, Student's t test).

Examination of the effect of systemic administration of tetracycline on LPS-induced subcutaneous inflammatory lesions in mice also revealed a marked positive effect. Subcutaneous injection of 0.1 mg of LPS into mice induced a visible lesion (60 to 80 mm^2) within 24 h, with tissue necrosis which started to heal spontaneously after 1 week (Fig. 2A and B). Daily administration (i.p.) of tetracycline for the first 4 days following LPS challenge reduced the size of the lesion in a dose-dependent manner (Fig. 2A and C). An optimal effect of tetracycline was seen at a dose of 75 mg/kg of body weight, with total inhibition of lesion formation occurring in 75 to 100% of the treated mice ($P < 0.01$, Z test and one-way analysis of variance). When lesions did occur, their appearance was delayed until the fourth day after LPS challenge, and they were markedly reduced in size (<10 mm^2) and lacked necrosis (Fig. 2A and B). Administration (i.p.) of 100 mg of tetracycline per kg abolished all signs of inflammation but resulted in up to a 20% lethality rate. A unrelated broad-spectrum antibiotic, ampicillin (25 to 100 mg/kg), had no effect on the lesion size (data not shown).

In order to explore some aspects of the mechanism involved, we tested the effect of tetracycline on the secretion of TNF-α and IL-1β by LPS-stimulated human monocytes. Tetracycline was found to inhibit LPS-induced TNF-α and IL-1β secretion into the culture medium in a dose-dependent manner (Fig. 3). Complete inhibition of the secretion of these cytokines was observed with 0.5 mg of tetracycline per ml, and the 50% inhibitory dose was ≈0.1 mg/ml. No cellular toxicity of tetracycline was observed at the tested concentrations. However, when the effect of tetracycline was tested at the mRNA level, no effect of tetracycline on IL-1β mRNA accumulation could be demonstrated in LPS-stimulated monocytes (Fig. 3C). The same results were obtained for TNF-α mRNA (data not shown).

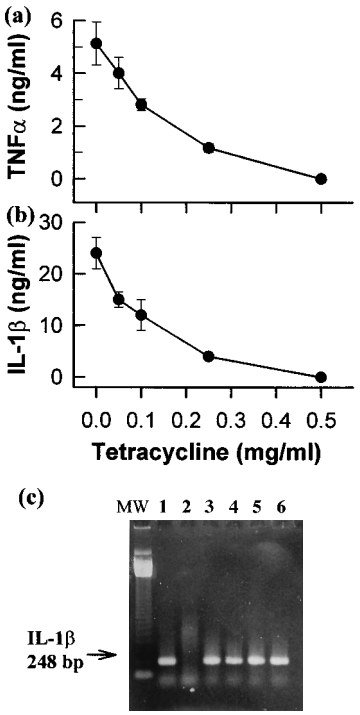

FIG. 3. Dose-dependent inhibition of LPS (1 μg/ml)-induced secretion of TNF-α (a) and IL-1β (b) from human monocytes by tetracycline. Cytokine secretion into the medium was quantified by ELISA. (c) Effect of tetracycline on LPS (1 μg/ml)-induced IL-1β mRNA accumulation in human monocytes. Lane MW, DNA length markers; lane 1, LPS-stimulated monocytes; lane 2, unstimulated monocytes; lanes 3 to 6, monocytes stimulated with LPS and 50, 100, 250, or 500 μg of tetracycline per ml, respectively. mRNA was semiquantified by reverse transcription-PCR.

DISCUSSION

There is little doubt that excess levels of inflammatory mediators lie behind the clinical manifestations and mortality associated with septic shock. Therefore, any intervention that inhibits the release of cytokines or neutralizes their effect is believed to benefit patients experiencing septic shock. The present study showed that treatment with tetracycline modulates the response to LPS and markedly reduces its lethal toxicity in mice. In addition, tetracycline was found to inhibit TNF-α and IL-1β secretion from human monocytes in vitro as well as to reduce serum TNF-α levels in vivo. TNF-α and IL-1β are thought to be central mediators in septic shock, and studies using animal models have shown that targeting TNF-α or IL-1β prevents the clinical manifestation of septic shock (1, 4, 5, 21, 23, 34, 41, 47, 48, 51, 55). However, studies using anticytokine strategies in humans were not as successful at saving patients' lives (3). It is reasonable to suggest that more than one mediator is involved in the pathogenesis of septic shock, and targeting a single cytokine might not be as effective as blocking several involved cytokines. The results presented here show that tetracycline inhibits the secretion of both TNF-α and IL-1β from human monocytes. These two mediators were found to be synergistic in the induction of a shock-like state (13, 32, 49). The oral doses of tetracycline that were used in the present study are severalfold lower than the reported 50% lethal dose for tetracycline (16), and no signs of toxicity were detected after oral administration. However, the i.p. injection of high doses of tetracycline (≥2 mg per animal) caused peritoneal injury and death in up to 20% of the animals. It was for this reason that we reverted to the oral administration of tetracycline.

LPS-induced secretion of cytokines from monocytes involves specific intracellular signal transduction events. Several studies show the involvement of protein kinase C, protein tyrosine kinase, and mitogen-activated protein kinase in this process (11, 12, 38, 42, 53, 54). Another step involved in TNF-α secretion is the proteolytic cleavage of membrane-anchored TNF-α and the release of the soluble form of this cytokine (29). This step is dependent on the activity of a specific metalloproteinase. In addition to having antibacterial properties, tetracycline has been shown to inhibit both protein kinase C and metalloproteinase activities (17, 52). The inhibition of these activities was found to be related to the strong chelating ability of tetracycline. It is therefore reasonable to hypothesize that these properties of tetracycline lie behind the mechanism by which cytokine secretion is blocked. The finding that tetracycline inhibits cytokine secretion but not cytokine mRNA accumulation suggests that the mechanism involves the blockage of posttranscriptional events rather than early intracellular events.

Tetracycline has been widely used to treat several localized inflammatory diseases, such as chronic acne and periodontal disease. However, the mechanism of its action is still unclear. It has been suggested that tetracycline is effective at least in part because of its inhibitory effect on tissue collagenase (17). Inhibition of cytokine secretion is another possible pathway by which tetracycline may function in these clinical situations. Indeed, high levels of TNF-α and IL-1β have been associated with tissues affected by periodontal disease, and these cytokines have been implicated as central mediators in the localized destructive process associated with this disease (40, 43).

In conclusion, tetracycline was found to inhibit TNF-α and IL-1β secretion from LPS-stimulated monocytes, to reduce LPS-induced serum TNF-α levels in vivo, to protect mice from experimentally induced septic shock, and to reduce the size of LPS-induced subcutaneous lesions. The data presented here suggest that the use of tetracycline or tetracycline derivatives might be an effective means of therapy in LPS-induced pathologies such as septic shock.

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